FACTORS INFLUENCING DEVELOPMENT OF META-CHROMATIC GRANULES IN THE DIPHTHERIA BACILLUS

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A study was made of some of the outside influences which may affect the production of granules in the diphtheria bacillus. Factors were sought that might hasten the appearance of granules and make an earlier diagnosis possible, and also those which might delay the appearance of the granules, thus interfering with the accuracy of routine examinations.

Types of granules were studied exclusively as most commonly seen in this locality. It is reasonable to suppose that conditions modifying granule formation would also modify the formation of bars in that type of organism.

The appearance of granules at different ages of the organism has been studied by Denny,[1] Albert[2] and others, who have found that granules appear in cultures from 4 to 8 hours old, and attain their largest average size in cultures from 12 to 15 hours old.

The effect of reaction of the medium on granule formation was studied by Layborne[2] and Bunker.[3] Layborne found that blood serum of P_H 7 to 7.5 gave the largest percentage of granule forms. As the reaction approached P_H 5.5 and 8.5 the organism became smaller and the percentage of granule forms less.

Denny[4] says that the transition from the solid to the granular form depends on either the accumulation of bacilli or products of growth of the organism. Denny also studied the effects of various incubation temperatures in reference to granule formation and found that the appearance of granules was delayed at a temperature of 19 to 21 C., and at a temperature of 40. He also found that symbiotic growth with various organisms delayed the granule production.

Heineman[5] and Mellon[6] varied the morphology of the organism, producing a coccus-like form from a granule form by growing it on veal glucose broth and bringing it back to the granule form on blood serum or blood agar.

Wherry[7] studied the morphology of one culture in relation to oxygen tension. He found that the barred and granule forms disappeared when grown on Loeffler's blood serum under anaerobic conditions and also that growth with B. subtilis produced the same effect.

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1 Jour. Med. Research, 1903, 9, p. 117.
2 Absts. Bacteriol., 1921, 5, p. 14.
3 Ibid., 1917, 1, p. 31.
4 Public Health, 1902, 28, p. 471.
5 Jour. Bacteriol., 1917, 2, p. 361.
6 Ibid., p. 81.
7 Jour. Infect. Dis., 1917, 21, p. 47.

Browning[8] states that the appearance of granules is due to either the method of fixation of the smear or the wash water used in making the preparation. This theory did not need to be considered since granules in this general group can be stained while the organisms are living.

Meader[9] states that the type may vary because of acid production from glucose.

Arloing and Richard[10] used various mediums, coagulated guinea-pig serum, Costa lecithin medium with beer yeast and phosphorus compounds and found that granules were produced.

SOURCE OF CULTURES

The cultures used were selected from a list of eleven. Nine of these were isolated from routine throat cultures obtained from the diagnosis division of the Municipal Laboratory. One was supposed to be Park 8 and was obtained from Lakeside Hospital. The remaining culture was from a case of clinical diphtheria at Mt. Sinai Hospital. Of these cultures, two were from single cell isolations. All cultures were virulent. These cultures were all good granule forms with the exception of Park 8, which was found to be variable in its granule production and was not used in the later experiments.

TECHNIC

All work was as carefully controlled as possible. On plates, when it could be done, one half of the plate was used as the control and the other half for the experimental work. In making preparations for morphologic study care was taken to obtain portions from as near the same part of the culture as possible.

The staining used was that of Albert,[11] except in the earlier work in which his first formula was used. It was the impression that Albert's stain was much more satisfactory for granule demonstration than Loeffler's methylene blue or some of its various modifications which were tried. To exclude possible errors in staining technic the controls were stained on the same slide and at the same time as the organism in the experiment. In most cases a count of 100 cells was made and the cells classified as: (1) granules; (2) unevenly stained cells, including organisms in which part of the cell showed a degree of staining different from the remainder but no definite granule formation; and (3) solid forms. When an examination of a preparation showed that the organisms were practically all of one type, a count

[8] Applied Bacteriology, 1918, p. 58.

[9] Jour. Infect. Dis., 1919, 24, p. 145.

[10] Bull. de l'Inst. Pasteur, 1921, 19, p. 738.

[11] Jour. Am. Med. Assn., 1921, 76, p. 240.

was not made, and the general impression of the type was given. Detailed tabulations of the results of counting are not given, and in the three tables the results of counting a number of cultures have been averaged in order to show in a condensed form the approximate granule, unevenly stained and solid forms.

PRESENCE OR ABSENCE OF CERTAIN NUTRITIVE MATERIAL IN THE MEDIUM

The incubation time was about 16 hours in all cases. A serum agar medium made after the manner of Klein,[12] but with ox blood serum in place of horse serum, was used extensively. The number of granule forms and the number of granules in each organism on this medium are about the same as on Loeffler's blood serum, although the organisms themselves are for the most part smaller in size. As Loeffler's blood serum contains dextrose while the Klein medium does not, 1% dextrose was added to Klein medium. The number of granule forms and the size of the organisms were the same as the Klein medium without dextrose.

Other mediums which gave a percentage of granule forms almost equal to that obtained on Klein medium were: blood serum digested with trypsin until it would not coagulate on boiling and then added to agar; Frost medium;[13] ordinary laboratory milk medium; serum water; a mixture of equal parts of milk and serum water; slants of egg yolks and egg white; Whittaker's [14] casein agar.

Mediums which gave a morphologic form and granule production similar to that seen on glycerol agar were Ayers and Mudge [15] milk powder medium; Huntoon's [16] hormone medium; Hitchens' lower percentage agar medium [17] and potato slants. Eberson's yeast medium [18] produced a peculiar coccus-like form with no granules. Gelatin produced few granule forms. Four cultures were grown on the following mediums with indicated results; the counts in each case are averaged:

Agar + peptone + meat extract = granule forms, 66; unevenly stained, 6; solid, 26.
Agar + peptone = granule forms, 8; unevenly stained, 12; solid, 79.
Agar + meat extract = granule forms, 31; unevenly stained, 19; solid, 50.

[12] Deutsch. med. Wchnschr., 1920, 1, p. 297.
[13] Jour. Am. Med. Assn., 1921, 76, p. 13.
[14] Jour. Am. Med. Assn., 1912, 37, p. 82.
[15] Jour. Bacteriol., 1920, 5, p. 565.
[16] Jour. Infect. Dis., 1918, 23, p. 169.
[17] Ibid., 1921, 29, p. 390.
[18] Jour. Am. Med. Assn., 1919, 72, p. 852.

Comment.—Agar with peptone gave in all four cultures a peculiar short almost ovoid organism. This was thought at first to be a contamination, but transplants on other mediums showed pure cultures to be present. A much higher percentage of granule forms is seen in the medium with meat extract than with peptone alone, but in neither case is the percentage as high as where both were used.

From these experiments it can be seen that:

More granule forms are seen when the basis of the medium is a complex protein nature such as blood serum in its various forms or that of egg or milk protein.

The protein derivatives found in meat extract give better results than those found in peptone when added to a plain agar base.

AMOUNT OF WATER IN MEDIUM AND GRANULE FORMATION

Difco desiccated blood serum was used in these experiments and when made as directed contained about 92% water. Eight cultures were inoculated on mediums made in this manner as controls. The same 8 cultures were then inoculated on the blood serum mediums made with varying amounts of water. The incubation time in all cases was 16 hours at 37 degrees.

The results obtained are given in table 1. In each case figures represent the average of the count of granules, unevenly stained and solid forms of the 8 cultures used.

A marked diminution in the number of organisms showing granules occurred in the mediums containing 80% and 70% water compared to the control containing 92% water.

The size and shape of the organisms and the number of granules per organism in the 80% of water were about the same as in the controls. In the 70% water mediums the organisms were slightly shorter. The results with mediums of less than 60% were inconclusive as the granule forms present were practically all of the normal blood serum morphology which were thought to be the remains of the mature granule forms of the transplant.

In view of this possibility, 6-hour growths of the same cultures on ordinary blood serum were inoculated on mediums of 50%, 40%, 30% and 20% water and incubated for 16 hours. The 6-hour cultures were used as controls.

The results are shown in table 2. As would be expected, there were few granule forms in the 6-hour cultures. When these were transferred to the mediums of small water content there was evidence

that some division had taken place in the increased number of young forms. Transplants from these cultures showed growth in several instances.

INFLUENCE OF METABOLIC PRODUCTS OF THE ORGANISM ON GRANULE FORMATION

As Denny had made the statement that granule formation depends on either the accumulation of the organisms or the products of the growth of the organism, this relation to crowding was studied in some detail.

Granule formation of organisms from single and confluent colonies was observed. Eight cultures were streaked on blood serum

TABLE 1

AMOUNT OF WATER IN THE MEDIUM AND GRANULE FORMATION FROM 24-HOUR CULTURES

	92% Water	80% Water	70% Water	60% Water	50% Water	40% Water	30% Water	20% Water
Granules	71	53	41	27	13	20	24	22
Unevenly stained	10	12	12	18	12	19	10	7
Solid	18	35	47	53	75	61	65	70

TABLE 2

AMOUNT OF WATER IN THE MEDIUM AND GRANULE FORMATION FROM 6-HOUR CULTURES *

	92% Water	50% Water	40% Water	30% Water	20% Water
Granules	14	5	6	3	5
Unevenly stained	23	11	9	6	10
Solid	63	83	84	90	85

* Count of 6-hour growth on blood serum with 92% water. The other counts are from 16-hour growths.

plates, Klein medium plates and glycerol agar plates so that there was a heavy growth on one portion of the plate and isolated colonies on another. Plates were incubated for 16 hours at 37 degrees. Preparations were made from single and confluent colonies and counts made and averaged in each case (table 3).

In each medium the granule formation was much more marked in the confluent than in the single colonies.

The question whether the growth products are able to pass through a thin film of agar and influence granule production was investigated. Glycerol agar plates with mediums less than 2 mm. in thickness were heavily inoculated with cultures and incubated for 24 hours. With a sterile razor blade, sections were lifted from these plates and inverted

into sterile Petri dishes. The uninoculated side of each was tested for sterility, and cultures streaked on the sections. Controls were made on glycerol agar plates. The plates were incubated for 8 and 16 hour periods. The counts tabulated in table 3 are the average of 8 cultures in each case.

The results show that in the 8-hour cultures there was a marked increase in the number of granule forms on the inverted sections when compared to the controls. On all inverted sections the predominant type was a short rod with 1 and occasionally 2 granules, the type usually expected in a 16-hour agar growth. The predominant type in the controls was the solid Hofmann or short rod which would be expected. In the 16-hour growths the number of granule forms on the inverted sections was practically the same as on the control medium and also about the same as the number found on the inverted section after 8 hours' observation.

TABLE 3

GRANULE FORMATION IN SINGLE AND CONFLUENT COLONIES

METABOLIC PRODUCTS PASSING THROUGH THIN FILM OF AGAR

	Blood Serum		Klein		Glycerol Agar		8 Hours Inverted Sections of Agar		16 Hours Inverted Sections of Agar	
	Single	Confluent	Single	Confluent	Single	Confluent	Control	Inoculated Section	Control	Inoculated Section
Granules	17	78	2	86	6	69	15	67	66	53
Unevenly stained	17	9	9	8	14	14	12	9	7	11
Solid	65	13	88	5	79	17	72	24	27	36

The metabolic products of the organisms can thus apparently pass through a thin film of agar and hasten granule production. This effect, however, is seen only in the young (8-hour) cultures. In the older cultures (16-hour) it is not seen, possibly because by this time the inoculated organisms have produced a sufficient quantity of these products so that there is present the maximum number of granule forms possible under the conditions of growth.

The products of organisms killed by moderate heat were utilized for effect on granule formation. Slant agar growths of 8 cultures were killed by moderate heat and then incubated for 3 weeks to allow autolysis to take place. Preparations from these cultures at this time showed organisms of typical agar morphology. Subcultures gave no growth so it was evident that little or no autolysis took place, and this method of obtaining products of the organism had to be abandoned.

Broth filtrates of several organisms obtained after 3, 10 and 13 days' growth were painted on one side of a divided glycerol agar plate after both sides of the plate had been inoculated with the homologous organism and other strains. Plates of glycerol agar, Klein medium and blood serum were used. The untreated side of the plate was used as the control. Eight and 16-hour observations were made, but in no case was there any effect of the filtrate on granule production.

A 24-hour growth of one broth culture was filtered and painted on glycerol agar plates in like manner. The results in this case were doubtful. The organisms from which the filtrate was made showed no increase in granule forms when bathed in the filtrate. Six of the 7 other cultures used did show a slight increase in granule forms in an 8-hour period.

A filtrate heated to destroy toxin, a dilute Schick toxin and a toxin-antitoxin used in the same manner showed no effect on granule formation.

The reaction of the broth was little changed by the growth of the organisms. The broth used had a reaction of P_H 7.4. The reaction of the 24-hour filtrate was between 7.4 and 7.5, and that of the 13-day filtrate was 7.7. Due to this slight change it is improbable that the reaction of the growing culture has any marked influence on granule production.

INFLUENCE OF TEMPERATURE AND GRANULE FORMATION

Eight cultures were used on glycerol agar and Klein medium. These were incubated for 16 hours at various temperatures. The results obtained by counting the cells and averaging the results may be summarized:

Glycerol agar cultures at 27 C. showed a reduction to 56% granule forms from the 73% produced at a temperature of 37 C. At a temperature of 18.5 C. the percentage of granule forms was 20%, and these were for the most part of the morphology of the cultures on Klein medium from which the transplants were made.

Klein medium slants at 37 C. showed 70% granule forms to be present, and at 21 C. there were 27% granule forms. Denny states that a temperature of 40 C. delays granule formation, so a higher than body temperature observation was not used.

OXYGEN TENSION

Eight cultures were grown on 1% dextrose glycerol agar and on Klein medium as controls. In tubes of similar cultures the upper half

was heated for 10-20 seconds and quickly corked with rubber stoppers. The cultures were incubated for 18 hours at 37 degrees.

The results obtained by counting the cells showed that this method of reducing oxygen did not influence granule formation.

Eight cultures grown anaerobically on the same medium showed a slight diminution in the number of granule forms in the dextrose agar and a marked diminution in Klein medium. In the latter medium there was an average of 75% of granule forms in the control and an average of 8% under anaerobic conditions. In the dextrose agar under anaerobic conditions the typical organisms were slightly elongated and coccus-like instead of the short rods containing one or two granules of the control. In the Klein medium the organisms were shorter than in the control but did not show this coccus-like form.

The 8 cultures, when grown at room temperature (18 C.) for 16 hours, under a vacuum which varied from 10 to 19 inches of mercury, showed no reduction in number of granule forms.

SUMMARY

Granules in the diphtheria bacillus are produced in a large percentage of organisms when the culture is grown on a medium of which the base is blood serum. The granules are produced in lesser numbers when grown on glycerol agar. The meat extract in glycerol agar apparently favors the production of granules more than does the peptone. But more granules are produced when both are used. A semiliquid nutrient agar medium does not favor granule formation.

The full number of granule forms develop in a blood serum medium containing 92% of water. When the amount of water is decreased below this amount there is an appreciable decrease of granule forms.

There is a marked decrease in the proportion of granule forms to others when smears are made from a single colony, as compared with smears from confluent colonies.

Products of growth of the organism can apparently pass through a thin film of agar and hasten the granule forms in young cultures. This does not hold true in older cultures.

A broth filtrate of an organism probably does not influence granule production either in that organism or other organisms. Neither diphtheria toxin, heated filtrate nor toxin-antitoxin influence granule formation.

Granule production is better at a temperature of 37 C. than at lower temperatures. Unless the temperature fell below 27 C. the effect was small.

Reduced oxygen tension does not influence granule production unless sufficient to absorb oxygen appreciably from the culture, then few granule forms are produced.

CONCLUSIONS

Denny's observation that the granule appearance depends on either the accumulation of bacilli or the formation of products of the bacilli is confirmed in these observations. The fact that there was an apparent stimulus when it was possible for the products to pass aseptically through a film of medium is most suggestive of the presence of metabolic products, but the lack of success with filtrates from fluid mediums indicates need of more work along this line. The observation on increased granule formation in crowded cultures is suggestive of the same point.

To produce granules in maximum quantity and at maximum speed, diphtheria organisms require suitable mediums, a large amount of water, a growth temperature close to 37 C., a certain amount of oxygen, and the accumulation of large numbers of organisms.